Carotid Artery Disease in Post-Stroke Survivors and Effects of Enriched Environment on Stroke Pathology in a Mouse Model of Carotid Artery Stenosis

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Abstract

Aims: Carotid artery disease (CAD) is an important risk factor for stroke. We first evaluated CAD and stroke pathology in elderly post-stroke survivors. To simulate CAD, we assessed long-term consequences of bilateral common carotid artery stenosis (BCAS) in mice and exposed them to environmental enrichment (EE).

Methods: Histopathological methods were used to determine degrees of CAD (% area stenosis), brain infarct types, sizes and distribution in post-stroke survivors and BCAS mice. Adult male C57BL/6J mice after BCAS or sham surgery were randomly assigned to standard housing (Std) or limited (3hrs) or full-time (Full) exposure to EE per day for 12 weeks.

Results: High frequencies of moderate carotid artery stenosis (51-75%) were evident in post-stroke survivors whereas those with severe CAD (>75% stenosis) exhibited greater numbers of cortical rather than subcortical infarcts and, were at higher risk of developing dementia. BCAS in mice reduced cerebral blood flow by 52% (P<0.01) and thickened carotid artery walls, regardless of EE duration. Remarkably, the total and cortical infarcts declined by >50% in BCAS mice exposed to EE compared with BCAS-Std (P<0.01). Frontal lobe and cortical strokes were associated with worsening working memory tested in a radial maze paradigm. Proteomic analysis revealed EE, both BCAS-3hrs and BCAS-Full attenuated coagulation cascade factors including fibrinogen and von Willebrand factor, markers of blood-brain barrier damage.

Conclusion: Small cortical and subcortical infarcts were evident in both post-stroke survivors with CAD and BCAS mice. Experimental evidence suggested that moderate exposure to EE is sufficient to reduce subsequent stroke lesions.

Keywords: carotid artery disease, cerebrovascular pathology, dementia, experimental model, stroke, environmental enrichment, post-stroke dementia, vascular brain injury, vascular dementia

Introduction

Carotid artery disease (CAD) is one of the most important risk factors for ischaemic stroke [1, 2]. Carotid intima-media thickness is used as a non-invasive ultrasound marker of early atherosclerosis, which is positively and robustly related to strokes [3]. CAD or carotid artery stenosis is also associated with markers of cerebral small vessel disease (SVD) such as white matter changes, lacunar and non-lacunar infarcts [4-7], greater mortality rates and recurrent strokes [8, 9]. However, it is not clear how much cross-talk there is between extracranial large artery disease and intracranial SVD responsible for cerebrovascular pathology [10]. It is possible that CAD causes the type of tissues changes, which are wholly attributable to intracranial SVD.

Environmental enrichment (EE), physical exercise and environmental stimulation have been suggested as a strategy to enhance motor recovery from stroke in post stroke patients [11, 12]. EE has been extensively explored in rodent experimental models of stroke injury [13, 14]. These studies showed that EE preserved motor and cognitive functions after stroke and enhanced neurogenesis. However, there are no studies to our knowledge that have explored the effects of differing degrees of EE on the consequences of CAD on recurrent events or cerebrovascular pathology, which is a common occurrence in the general population.

With the aims of identifying stroke pathology and practical interventions in individuals with CAD, we first assessed the type and extent of stroke lesions present in elderly post-stroke survivors with varying degrees of CAD, stratified according to current radiological criteria [15, 16]. To simulate cerebral effects of CAD in the general absence of ageing-related SVD, we quantified stroke pathology as a consequence of carotid artery occlusion in adult mice with long-term bilateral common carotid artery stenosis (BCAS) [17-20]. We used two different paradigms of limited (BCAS-3hr) and full-time (BCAS-Full) exposure to EE to assess subsequent stroke injury and cognitive function in BCAS mice.

Materials and Methods

Stroke Subjects

We assessed post-stroke cases from the Cognitive Function After Stroke (CogFAST) study [21, 22]. Briefly, first time ischaemic stroke patients >75 years old were enrolled into the study 3 months post-stroke to enable resolution of acute post-stroke delirium with a

standardized battery comprised of medical history, MMSE score, assessment of neurological deficits and activity of daily life (ADL), a blood screen, review of vascular risk factors and CT brain scan undertaken at the time of the stroke. We used the Oxford Handicap Scale to determine functional outcomes in relation to ADL [23, 24]. Stroke survivors were diagnosed with post stroke dementia (PSD) or designated as post-stroke no dementia (PSND) [22]. Ethical approval and permissions for this study using donated human brains was granted by the Newcastle and North Tyneside 1 Research Ethics Committee and facilitated by the Newcastle Brain Tissue Resource (NBTR).

Assessment of Carotid Artery and Cerebrovascular Pathology

Demographic details of the 70 post-mortem cases are presented in Table 1. In the majority of cases, the cause of death was bronchopneumonia. Brains and segments of carotid arteries at the internal and external bifurcation were retrieved at post-mortem. Brains were sampled bilaterally and assessed according to the Newcastle brain dissection protocol [25]. Macroscopic and microscopic pathology was assessed using standardised protocols [21, 26]. Briefly, macroscopic infarcts were recorded by visual inspection during dissection, and their presence was subsequently confirmed by microscopy. The size and number of infarcts in both hemispheres in the cortex, white matter, basal ganglia, thalamus, brainstem and cerebellum was recorded as follows: <5 mm, 5-15 mm, 16-30 mm, 31-50 mm and >51 mm. Haematoxylin and Eosin (H&E) stain was used for neuropathological assessment including vascular pathology score [27] to assess SVD pathology (Supplemental information). Gallyas and Bielschowsky's silver impregnation and tau immunohistochemistry were used to assess neuritic plaques and neurofibrillary tangles for the 'CERAD' plaque score and 'Braak and Braak' neurofibrillary tangle staging. Pathological diagnosis of vascular dementia (VaD) was assigned, if there was clinical evidence of dementia (DSM IV) and relevant vascular pathology in the general absence of neurodegenerative pathology i.e. Braak staging <5 [25]. Severity of cerebral amyloid angiopathy (CAA) was assessed using four-point scale based as described previously [28]. Further details in Supplemental information.

Carotid artery stenosis was stratified into mild, moderate and severe categories based on modifications of the ultrasound and angiographic methods used in the European Carotid Surgery Trial (ECST) and the North American Symptomatic Endarterectomy Trial [15, 29]. Our moderate classification (% area) corresponded to low moderate whereas severe category

corresponded to both high moderate and severe in the ECST. In this manner, we could fit all our cases into 3 basic categories of mild (<50%), moderate (50-75%) and severe (>75%) stenosis. Samples of the right and left internal carotid arteries (ICAs) were taken at 4mm distal to the level of the carotid bifurcation. Ten-µm thick serial sections cut from paraffin embedded transverse ICA blocks were stained with H&E and used for quantitative analysis. Sections with the highest degree of stenosis were scanned using a photo scanner (EPSON Perfection V700). Total area of the external margin of the adventitia (S1) and luminal area at the interior margin of the intima (S2) were measured using Image J software. The % area stenosis was calculated using following formula: % area stenosis = (S1 - S2) / (S1) x100. The % area stenosis of both right and left ICAs were calculated and averaged. All data analyses were performed by at least 2 investigators blinded to case identities.

BCAS Surgery in Mice, Cognitive Behaviour and EE Assessment

Details of all procedures and cognitive testing paradigms in mice [17, 30, 31] are provided in Supplemental Information.

Measurement of Cerebral Blood Flow

Cerebral blood flow (CBF) was assessed by in-vivo imaging of in all mice using laser speckle flowmetry (MoorFLPI2, Moor Instruments, UK) at 16 weeks after surgery [30].

Mice Brain Tissues and Histopathological **analysis**

After euthanasia, the right or left hemisphere was randomly selected for histological analysis. Brains either post-fixed in 4% paraformaldehyde (PFA) in 0.01 mol/L PBS (pH 7.4) for 48 hours or fresh frozen for proteomic analysis (see below). For histopathological analysis, brains were cut into 5 blocks at different coronal levels as follows: Block OB, coronal level of olfactory bulb; Block 1, coronal level of bregma +0.5 mm; Block 2, coronal level of bregma -1.0 mm; Block 3, coronal level of bregma -2.0 mm; Block 4, level of cerebellum and brain stem. Each block was then embedded in paraffin and used to cut 5-µm thick sections for analysis.

Common carotid arteries (CCAs) were dissected from the exact or approximate location of the microcoils from BCAS and sham animals, respectively. Samples were post-

fixed in 4% PFA and cryoprotected in 20% sucrose in 0.6 mol/L phosphate buffer (PB). They were then protected with OCT compound (CellPath, UK) for freezing in liquid nitrogen-cooled isopentane prior to cutting 10-µm thick sections for staining and morphometric analysis.

CCA wall thickness and sclerotic index (SI)

H&E stained sections were used to assess arterial wall thickness and sclerotic index of CCA. Wall thickness was determined from 16 measurements encompassing the entire wall at 16 different levels at equal intervals in each CCA. For the sclerotic index (SI) [32], the internal and external diameters of each CCA was measured in four opposing axis. SI was then calculated using the formula: SI = (External diameter - Internal diameter) / (External diameter). All images were captured using a bright field microscope (Leitz DIALUX 20, Leica) with 20x objective lens coupled to a Lumenera infinity digital camera (Lumenera Corporation, Canada).

Incidence, Distribution and Volume of Strokes

Haematoxylin and Eosin (H&E) and Klüver-Barrera (KB) stained serial sections obtained from 3 blocks were used to determine stroke pathology in mice brains. Blocks were cut coronal levels from bregma +0.5 mm, -1.0 mm and -2.0 mm. The presence of ischaemic or haemorrhagic infarcts and microhaemorrhages was recorded in each mouse. We then determined the incidence (%), location and percentage of these lesions. To assess the stroke volumes in each mouse, the damaged area was traced using ImageJ software. The stroke area (mm^2) was calculated and multiplied by the thickness (mm) of each block. The stroke area (mm^2) x thicknesses (mm) from each brain block was added to calculate total stroke volumes in each brain as well as cortical and white matter stroke volumes (mm^3). Images were captured using a bright field microscope (Leitz DIALUX 20, Leica) with 5x, 10x and 20x objective lenses coupled to a Lumenera infinity digital camera (Lumenera corporation, Canada).

Quantitative Proteomic Analysis by TMT6-based LC-MS/MS

Brain tissues (~40 mg/group) including the white matter from BCAS and sham animals were retained for molecular analysis [33-35]. Details of the proteomic analysis using the TMT6-based LC-MS/MS are provided in Supplemental Information.

Database search and data analysis

Acquired data were processed using Proteome Discoverer v2.2 (PD2.2, Thermo Scientific, San Jose, USA). Details are provided in Supplemental information [36]. A total 95 % of the proteins identified in our study displayed a ratio % CV <50 %, therefore only proteins with % CV >50 were considered as differentially regulated. Furthermore, only those differentially regulated proteins, which showed a False Discovery Rate (FDR) adjusted p-value (q-value) <0.001 were considered.

***Statistical* analysis**

Data were expressed mean ± SEM. Using IBM SPSS statistics 23 software, distribution of data was analysed by Shapiro-Wilk test followed by Kruskal-Wallis H or Mann-Whitney U test for non-parametric analysis. One-way ANOVA or non-paired t-test was performed when data were normally distributed. Chi-square test was used to compare i.e. gender ratio, prevalence of dementia, vascular risk factors and CAA pathology in human cohort as well as incidence of stroke and distribution of stroke site and size in both human and animal groups. Spearman's correlation analysis was used to assess the relationship between cognitive function and infarct volume in the BCAS cohort. $P<0.05$ was defined as statistically significant in all analyses. Details of the box plots are provided in Supplemental Information.

Results

CAD, ADL and Cerebrovascular Pathology

Mean age and gender (women/men) ratios were not different between mild, moderate and severe ICA stenosis groups (Table 1). Of the 70 subjects we assessed, 17.1% exhibited mild stenosis, 61.4% had moderate stenosis and 21.4% had severe stenosis prior to death. The mean % area ICA stenosis was highest in severe cases (85.0%) compared to the moderate (64.5 %) and mild ICA stenosis subjects (44.1%) ($P<0.01$) (Figure 1a; Table 1). Mild stenosis subjects showed minimal intimal thickening. In addition to intimal thickening,

severe ICA stenosis subjects showed thick fibrous cap lesions (Figure 1a). Some severe ICA stenosis cases also showed calcified lesion and intra-plaque haemorrhage (data not shown). Seventy-five % of post-stroke subjects who developed dementia met the pathological criteria for VaD [22, 37]. The frequency of dementia was greater in both the moderate and severe ICA stenosis groups (P<0.01). ADL assessed by the Oxford Handicap Scale revealed that all mild ICA stenosis subjects exhibited no symptoms (17%) or minor symptoms (83%) not affecting lifestyle (P<0.01). On the other hand, greater percentage of more severe symptoms, e.g. minor handicap or moderate symptoms with impaired ADL were evident in patients with moderate and severe ICA stenosis groups, 21% and 40% in moderate and severe ICA stenosis group respectively (P<0.01) (Figure 1b).

We further evaluated the SVD, CAA pathology as well as distribution of stroke locations and sizes in the post-stroke subjects. Mean vascular pathology scores in post-stroke patients were consistently high, suggesting severe SVD pathology, regardless of severity of CAD (Table 1). Prevalence of moderate-severe CAA pathology was low (~20%) across all mild, moderate and severe ICA stenosis groups (Table 1). Furthermore, irrespective of the degree of carotid stenosis, we found that the greatest numbers of infarcts (range 43-63%) were in the cortex. In accord with the staging of carotid stenosis, we found the cortical infarcts were 45% (23 of 51) in the mild, 43% (75 of 175) in the moderate and 63% (40 of 64) in the severely stenosed groups (Figure 1c). The highest number being in the severe stenosis group (P<0.01). The basal ganglia and thalamus contained the second highest numbers of infarcts (range 20-31%) (Figure 1c) with the slightly greater numbers in the mild and moderate stenosis groups compared to the severe stenosis group. These were 20%, 31% and 22% in mild, moderate and severe stenosis groups, respectively). Approximately 13% of the total infarcts were present in the white matter. The brainstem had the least numbers of strokes (Figure 1c). In terms of the size, by far the majority of stroke lesions were small, < 5mm (range 74-76%). The mild and moderate stenosis groups had greater numbers of stroke sizes 5-15 mm than the severe group. Thus, mild and moderate severe stenosis groups tended to have the smallest infarcts (Figure 1d). Haemorrhagic changes including microbleeds in cortical and subcortical regions were observed in 11% of the CAD patients. However, only one case in the moderate ICA stenosis group showed several cortical microhaemorrhages with related CAA (grade 3) pathology.

CBF after long-term BCAS in mice

CBF values were standardised to the Sham-Std group. Mean CBF values of sham subgroups were: Sham-Std, 100%; Sham-3hrs, 106.3%; Sham-Full, 98.5%. At 16 weeks after surgery, CBF was reduced to 47.8% in the BCAS-Std subgroup compared with all sham subgroups (P<0.01). BCAS-3hrs subgroup showed the least reduction of CBF to 59.2%, followed by CBF reduction in BCAS-Full of 56.8% (both P<0.01 compared with all sham subgroups) (Figure 2a and b). There was no significant difference in CBF reduction between BCAS subgroups, however, the BCAS-Std subgroup showed the lowest CBF, and limited exposure to EE (BCAS-3hrs) group showed the most preserved CBF at 16 weeks after BCAS surgery (Figure 2a and b).

Effects of BCAS on Common Carotid Arteries

Upon gross examination of the CCAs, we observed low-grade fibrosis around vessels but no evidence of occlusion/stenosis in sham animals. All mice with BCAS showed corroded microcoils in a similar manner. Inflammatory changes, fibrosis and post-operative adhesion were prominent around CCAs in the BCAS animals. The distal side of CCAs were collapsed indicating arteries were either entirely occluded or severely stenosed. BCAS caused artery changes characterised by thickened walls, increased sclerotic index (SI) and greater % area stenosis mainly due to intimal thickening compared to that of Sham animals (Figure 2 c-f), regardless of the length of exposure to EE. Sham animals did not exhibit any apparent pathological arterial wall changes. Carotid arterial wall thickness was increased by >2.3-fold in BCAS animals with mean width of 23.8 ± 1.7 μm in sham and 53.8 ± 3.1 μm in BCAS animals (P<0.01) (Figure 2d). Mean SI was also increased by nearly 2-fold with a value of 0.20 ± 0.01 in sham and 0.37 ± 0.02 in BCAS animals (P<0.01) (Figure 2e). BCAS showed 1.5-fold greater CCA stenosis with mean % stenosis of 64.0 ± 2.8% compared to Sham of 42.5 ± 0.9% (Figure 2f). EE did not have significant effects on carotid artery wall thickness, SI or % area stenosis (Figure 2 g-i).

Infarction after BCAS: Limited versus full-time EE

In the intervening period of 16 weeks post-surgery, BCAS-Std induced stroke injury in 100% of the mice (Figure 3a; Table 2). While it was difficult to observe overt neurological deficits in the live mice, stroke pathology within the brains included ischaemic and haemorrhagic infarcts and microhaemorrhages. Again, as in post-stroke survivors, the majority (46%) of the

infarcts were in the cortex with 21% in the caudoputamen (Figure 3b). Remarkably, the incidence of infarcts was reduced by 40% in the mice with limited exposure to EE (BCAS-3hrs) and by 33.3% in the BCAS-Full mice (both $P<0.01$) (Table 2). Also, the incidence of cortical infarcts was reduced by 61.8% in the mice with limited exposure to EE (BCAS-3hrs) and by 31.8% in the BCAS-Full mice (both $P<0.01$) (Table 2). Furthermore, the incidence of frontal cortical infarcts was reduced by 21.7% in the mice with limited exposure to EE (BCAS-3hrs) compared with BCAS-Full and by 18.5% compared to the BCAS-Std (both $P<0.01$) (Table 2). Limited exposure to EE also reduced the total percentage of both cortical and subcortical (caudoputamen) infarcts after BCAS compared to BCAS-Std and BCAS-Full subgroups ($P<0.01$) (Figure 3b). Stroke pathologies found in the thalamus/hypothalamus in BCAS subgroups were of the haemorrhagic type. In caudoputamen, BCAS-Std subgroup showed 30% ischaemic infarcts and 70% of haemorrhagic infarcts, whilst in BCAS plus EE subgroups all infarcts had evidence of haemorrhages (Figure 3a). In terms of the volume of stroke, BCAS-3hrs group had greater numbers of stroke sizes <0.15 mm^3 than the BCAS-Std and BCAS-Full groups. Thus, BCAS-3hrs group tended to have smaller infarcts compared with BCAS-Std and BCAS-Full groups ($P<0.01$) (Figure 3c).

Unlike in post-stroke survivors where we were limited in sampling the whole brain [25], we determined infarct volumes in brains of BCAS mice. Total infarct volumes after BCAS were reduced by 71% in limited (3hrs) ($P<0.05$) and by 64% in full-time exposure to EE compared with BCAS-Std. Limited exposure to EE subgroup showed the least volume of total infarction (Figure 3d). Cortical infarct volumes were reduced by 72% in limited exposure to EE ($P<0.05$) and by 63% in BCAS-Full compared with BCAS-Std (Figure 3e). Although infarct volumes in the frontal lobe and frontal cortex were not significantly different between BCAS subgroups, BCAS plus limited exposure to EE (BCAS-3hrs) exhibited the smallest volume of infarction (68-69% reduction compared with BCAS-Std) (Figure 3 f and g). White matter infarct volume showed trend toward less infarct volume in the BCAS-3hrs amongst BCAS subgroups. There was 50% reduction compared with BCAS-Std and 40% reduction compare to BCAS-Full.

Infarction after BCAS and Cognitive Behaviour

Working memory deficit assessed by 3D 9-arm radial maze characterised by low average arm repeat scores was evident in all BCAS animals compared to sham, ($P<0.01$). Between Sham

subgroups, average arm repeat score in sessions 11-20 was the highest in Sham-Full, indicating better cognitive function followed by Sham-3hrs and Sham-Std (Figure 4b). Amongst BCAS subgroups, arm repeat score averaged from sessions 11-20 in the BCAS-3hrs showed the highest mean score, indicating relatively preserved working memory, followed by BCAS-Full and BCAS-Std (Figure 4b). All BCAS subgroups showed lower arm repeat score compared with Sham subgroups, particularly with Sham-Full (All $P<0.01$) (Figure 4b).

Volume of infarction correlated with working memory deficit. Greater volume of infarction with working memory deficit was evident in BCAS-Std followed by BCAS-Full across BCAS subgroups. Limited exposure to EE (BCAS-3hrs) tended to show less infarct volume, particularly in the frontal lobe with relatively preserved cognition (Figure 4 c and d). Volume of infarct in the frontal lobe was negatively correlated with arm repeat scores (Spearman's rho= -0.38, $P=0.024$) (Figure 4c). Volume of infarct in the frontal cortex was also negatively correlated with arm repeat scores (Spearman's rho= -0.47, $P=0.004$) (Figure 4d). Moreover, total and cortical infarct volumes were negatively correlated with arm repeat scores (Spearman's rho= -0.35, $P=0.042$ and Spearman's rho= -0.36, $P=0.035$ respectively).

Evidence of Functional Protein changes after EE

Concentrating on the stroke phenotype, we found a number of peptides as signatures of proteins involved in microvascular pathology (Figure 5; Table 3). The abundance log2 ratio in all cases was compared to the Sham-Std animals. The restrictive cut-off range based on 50% CV was established to indicate significant regulation. We generated a list of 18 proteins with q-values (FDR adjusted p-values) lower than 0.001 (Table 3 and Supplementary Table). The potential role(s) of these differentially regulated proteins in the apparition/progression of brain infarcts was based on previous publications (Table 3). We were able to identify significant regulation of 8 proteins in Sham-Full and BCAS-Full compared to Sham-Std and BCAS-Std respectively (Figure 5). Except for calmodulin, we found that proteins associated with the coagulation cascades were increased in the BCAS-Std group with subsequent decreases in the BCAS plus EE groups. In particular, fibrinogen alpha chain, von Willebrand factor (vWF or Factor VIII) and Misato homolog 1 were the more regulated proteins at 3hr than full-time EE. They were significantly increased in BCAS-Std animals and then declined after limited EE but more so with full-time EE. Calmodulin, a protective protein against ischaemic stroke injury, increased during EE in Sham animals and was sustained at high

levels during BCAS-Std and BCAS plus EE. Other proteins related to hypoxic injury were more regulated after full-time EE compared to limited (3hr) EE (Table in Supplementary Information).

Discussion

Our study in elderly stroke survivors and in BCAS mice with pathologically verified carotid stenosis provides several novel findings. Foremost, there appear to be no large quantitative studies on CAD and stroke pathology in the same subjects [38]. We first showed that stroke survivors with severe CAD develop greater numbers of cortical rather than subcortical infarcts and that severe CAD is not necessarily associated with severity of SVD or CAA pathology or large infarcts. More than 70% of the stroke lesions in individuals with some degree of CAD were 5mm or less in size. This is consistent with numerous small infarcts or microinfarcts evident in patients who develop dementia [39-42]. Moderate to severe stenosis in the carotid arteries was also associated with greater risk of developing dementia and impaired ADL [23, 43].

Remarkably, in BCAS mice, albeit adult animals with significant carotid stenosis we found that cortical infarcts, predominantly microscopic, were the most prominent type of stroke injury. The development or recurrence of both cortical and subcortical infarcts were substantially reduced in number and volume by limited EE. We further demonstrated the worsening relationship between working memory as attested by impaired arm entries and frontal lobe or cortical stroke volumes. We also found functional evidence for the protective effects of limited and full time EE by the down regulation of coagulation cascade factors. In particular, markers to monitor blood- brain barrier (BBB) damage such as vWF and fibrinogen were attenuated in brains of BCAS mice exposed to EE compared to the BCAS-Std animals. This may implicate greater sealing of the BBB and relate to reduced infarction as a result of EE. Overall, our findings demonstrate the favourable effects of EE against stroke injury subsequent to long-term carotid artery stenosis in a mouse model of chronic cerebral hypoperfusion.

Although EE exposes animals to a number of different features e.g. physical activity, social interaction, vigilance and anxiety, we suggest the main effects of EE appear to be due to physical activity. The average number of wheel rotations in BCAS-Full subgroup was 5-fold greater compared with BCAS-3hrs, Sham EE subgroups. These findings collectively

convey a robust clinical message that even a limited amount of EE would be beneficial to stroke survivors to reduce the burden, prevent recurrent strokes, protect the BBB and improve or maintain cognitive status. This is consistent with previous studies in which physical exercise was demonstrated to be beneficial for motor and cognitive functions after stroke [12, 44-47]. We propose that EE in stroke survivors is implemented by 35-minute sessions of moderate physical exercise, cognitive training, and mindfulness practice [48]. This will have beneficial effects on reducing recurrent events and working memory function. Greater positive effects of physical exercise in man as opposed to mice is also likely because vascular risk factors would be strictly controlled after the first stroke to prevent recurrent events and further tissue injury.

Consistent with our previous findings cognitive function [49, 50] specifically working memory deficits were improved in BCAS animals exposed to limited EE [30]. It would seem lower volumes of frontal lobe and cortical infarcts in BCAS mice exposed to EE indicate reduced disruption of the frontal subcortical circuitry. However, the differential responses between the BCAS-3hrs and BCAS-Full may be explained by the oft-repeated initiation and activation of the frontal subcortical circuits in animals with limited exposure to EE. Effective activation of the frontal subcortical circuits could enhance cognitive function and prevent cognitive decline after cerebral hypoperfusion. Limited exposure to EE appears a safe and effective intervention for subjects with CAD or cerebral SVD in which perivascular oedema and thickening or disintegration of the arteriolar wall in subcortical structures are common [7]. We suggest in cases with smaller burdens or milder injury after stroke, there would be a better recovery after limited exposure to EE.

Histological analysis revealed that the CCA walls were thickened and SI and % area stenosis were increased in the BCAS group indicative of severely stenosed CCAs regardless of different types of EE. Similar CCA pathologies i.e. intimal hypertrophy coupled with gradual CBF reduction in both cortical and subcortical regions were reported in a mouse model of gradual carotid artery stenosis [51]. This fact supports our findings that BCAS-induced severely stenosed CCAs are a major cause of prolonged CBF reduction and precursors to the stroke injury. Notably, post-stroke patients with CAD represented similar severity of cerebrovascular lesions, related to the degree of % ICA area stenosis. Thus, long-term carotid artery stenosis may trigger stroke pathology and EE contributed to reduce stroke injury even in the presence of substantial carotid artery stenosis.

Although we have provided ample evidence of stroke pathology in both man and the mouse model of long-term carotid artery stenosis, actual causes of strokes remain to be elucidated. Haemorrhagic transformation indicates involvement of thromboembolic mechanisms, due to abnormal permeable blood-brain barrier (BBB) [52]. Artery-to-artery embolism due to carotid plaques and hemodynamic effects because of severe carotid artery stenosis are the two main causes of stroke injury in patients with CAD [53]. Other possibility is cardiogenic embolism due to atrial fibrillation (AF) [54]. In this study, we observed haemorrhagic transformation in post-stroke patients with ICA stenosis. Some of these haemorrhagic changes may have been caused by artery-to-artery embolisms due to CAD. We also noted ~20% patients exhibited clinical evidence of AF in life (Table 1). AF is one of the most important risks of cardiogenic embolism, which is known to cause haemorrhagic transformation. We suggest haemorrhagic cerebrovascular pathology detected in the post-stroke survivors is attributable to either artery-to-artery or cardiogenic embolism or both.

In the long-term BCAS model, chronic haemodynamic impairment is likely the cause of stroke lesions [55]. However, we also observed haemorrhagic cerebrovascular pathology in the caudoputamen and thalamus/hypothalamus, suggesting involvement of thromboembolic mechanisms [52]. Micro-emboli could be formed in either stenosed carotid arteries, which may cause artery-to-artery embolism, or in the arrhythmic heart. We previously reported hypertrophied heart muscle after long-term BCAS [30]. This heart condition may cause congestive heart disease and micro-emboli can be formed in heart due to low ejection fraction (EF). Therefore, haemorrhagic changes detected in the BCAS mice may also be attributable to thromboembolic mechanisms. However, histopathology did not reveal any thrombi in the lumen of stenosed carotid artery or in the heart, probably because mice were perfused when they were euthanised and thrombi might have been washed out from the body. While there was clear evidence for beneficial effects of exposure to EE, understanding of detailed mechanisms relating to limited and full-time exposure to EE against CAD and subsequent stroke injury remains unclear. However, the proteomic data suggest attenuation of coagulation cascade factors and modulation of protective factors such as calmodulin in CAD is possible. Also, we recently reported protein profiles in the brain-derived extracellular vesicle (EVs) were similarly regulated under a condition of cerebral hypoperfusion in both the human brain and long-term BCAS mouse brain [56]. Further application of in-vivo physiological screening e.g. vascular autoregulation, hemodynamic changes as well as assessment of angiogenesis, neurogenesis, neurovascular and gliovascular

integrity [57] would enable elucidation of mechanisms associated with effects of EE in long-term BCAS models.

There are some limitations of this study. First, we compared consequences of carotid stenosis on stroke pathology in ageing humans versus younger mice. While this allowed us to simulate the contribution of carotid stenosis *per se* in mice without complications of additional vascular risk factors or ageing, ideally aged mice may have been a better model. In a previous study comparing young and aged rats with middle cerebral artery occlusion [47], aged animals showed more severe behavioural impairments and much slower sensorimotor functional recovery compared to young rats. However, in accord with our data here in BCAS mice, EE improved the rate and extent of recovery even in the aged rats. Second, we could not test the effects of EE or physical activity in the post-stroke cohort as we would need to have set up an entire new study. Third, physiological and histological data obtained from several more time points would have been useful in understanding progressive pathological changes as well as effects of EE against carotid artery stenosis and subsequent stroke injury. Fourth, we implemented limited exposure to EE regime at 3 hours per day. Testing lesser duration of EE would perhaps define the optimal exposure to EE that would be most effective against vascular insults induced by carotid artery stenosis. Finally, although we suggested several possibilities based upon our findings, actual causes of strokes in long-term BCAS still remain to be elucidated. Further investigation is required to prove their pathogenesis.

In summary, long-term BCAS in mice produced similar stroke pathology characteristics to those observed in CAD in man. In this context, BCAS in mice is an appropriate model to explore the pathophysiology of small strokes. We also demonstrated that exposure to EE reduced strokes and elicited beneficial neurovascular effects of carotid artery stenosis. Moderate/limited compared to continuous exposure to EE appears a safe and effective interventional strategy against cerebrovascular diseases.

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Author Contributions

YH, RZ, MH, WS, AE, G-PX and RNK performed or contributed to different aspects of the experiments or analysis in the study. TMP and RNK performed the pathological examination and provided diagnosis. LA updated and provided the clinical databases. YH wrote the first draft of the manuscript. YH, MI, LA, KH, SKS and RNK contributed to critically revising the manuscript for important intellectual content, and all approved the final version of the manuscript for submission.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Carotid artery pathology, ADL and cerebrovascular lesions in post-stroke patients. a, Representative images of internal carotid artery (ICA) with mild (intimal thickening), moderate and severe stenosis (fibrous cap lesion) in post-stroke patients. † represents the lumen of stenosed ICA. Scale bar represents 1mm. **b,** Histogram showing distribution of the Oxford Handicap Scale in post-stroke patients with ICA stenosis [23]. Mild subjects had no symptoms or only minor symptoms with independent ADL, whereas a greater percentage of moderate and severe stenosis subjects showed more severe symptoms with impaired ADL (***P*<0.01). No subject showed moderately severe to severe handicap. **c,** Pie charts showing location of brain infarcts in subjects with mild, moderate and severe ICA stenosis. The majority of infarcts were observed in the cerebral cortex across all ICA stenosis subjects. The incidence of cortical infarction was higher in patients with severe ICA stenosis compared to those with mild or moderate ICA stenosis (***P*<0.01). **d,** Pie charts showing distribution of brain infarcts size in patients with mild, moderate and severe ICA stenosis. More than 74% of the infarcts were small (<5mm in size) regardless of the degree of ICA stenosis, and smaller infarcts, <15mm in size, were frequently detected particularly in mild ICA stenosis subjects.

Figure 2. Measurement of cerebral blood flow and common carotid artery pathology induced by BCAS. BCAS caused prolonged CBF reduction, thickening of walls in the common carotid arteries at 16 weeks after surgery regardless of different types of EE**. a,** Representative images of cerebral blood flow (CBF) obtained by laser-speckle flowmetry at 16 weeks after surgery. **b,** Box plot showing temporal profiles of averaged surface CBF in sham and BCAS subgroups. CBF was expressed as a ratio of the Sham-Std level. **P*<0.01 vs all Sham subgroups; †*P*<0.01 vs all Sham subgroups; ‡*P*<0.01 vs all sham subgroups. **c,** Representative images of the common carotid artery (CCA) in sham and BCAS groups. Scale bar represents 100 μm. **d-i,** Histograms showing wall thickness **(b and g),** sclerotic index (SI) **(e and h)** and % area stenosis **(f and j)** in Sham and BCAS groups. Wall thickness and SI values were significantly increased by 2-fold (***P*<0.01) and % area stenosis was greater by 1.5-fold (***P*<0.01) in the BCAS mice (**d-f**). No significant difference was seen between BCAS subgroups (**g-i**).

Figure 3. Stroke pathology induced by BCAS. Limited exposure to EE countered recurrent stroke injury more effectively than full-time exposure to EE**. a,** Representative images of cortical and subcortical infarcts observed in BCAS group. Black arrows indicate cortical infarcts and white arrows indicate subcortical haemorrhagic infarcts in the caudoputamen. Scale bars represent 200 μm. **b and c,** Pie charts showing location of brain infarcts **(b)** and distribution of brain infarcts volume **(c)** in each BCAS subgroup. BCAS plus limited exposure to EE (BCAS-3hrs) group showed a smaller percentage of cortical and subcortical (caudoputamen) infarct (in blue and orange, ***P*<0.01) compared with BCAS-Std and BCAS-Full. **c,** Pie charts showing distribution of stroke volumes in each BCAS subgroup. BCAS plus limited exposure to EE (BCAS-3hrs) group showed greater percentage of smaller infarcts, <0.15 mm^3 in size (in blue and red, ***P*<0.01) compared with BCAS-Std and BCAS-Full. **d-g,** Histograms showing total **(d)**, cortical **(e),** frontal lobe **(f)** and frontal cortical **(g)** infarct volumes in each BCAS group. **d,** Total infarct volume was smaller in both BCAS-3hrs (**P*<0.05) and BCAS-Full subgroups compared with BCAS-Std subgroup. **e,** Cortical infarct volume was reduced in BCAS-3hrs subgroup (**P*<0.05) compared with BCAS-Std, and showed a non-significant trend toward less infarct volume in BCAS-Full subgroup compared with BCAS-Std. **f and g,** BCAS plus limited exposure to EE (BCAS-3hrs) showed the smallest volume of infarction in the frontal lobe and frontal cortex (68% and 69% reduction respectively) compared with BCAS-Std.

Figure 4. Stroke Volumes and Working Memory Deficit after BCAS. BCAS caused cognitive decline (low arm repeat scores) and volumes of stroke were related to average arm repeat scores. **a,** Arm repeat scores and probability of each number of arm repeat in 9 arm entries. Arm repeat scores for each number of arm repeat were defined as the difference in percentage compared to the most probable number of arm repeat. Number of arm repeat more than 6 did not occur in this experiment (N/A). **b,** Histogram showing average arm repeat scores in sessions 11-20 in Sham and BCAS subgroups. **P*=0.003; †*P*=0.034; ‡*P*=0.003 vs Sham-Full. **c and d,** Dot plots showing correlation between arm repeat scores and volume of infarction in the frontal lobe **(c)** and frontal cortex **(d)**. Spearman's correlation analysis revealed that arm repeat scores in BCAS animals exhibited negative correlation with volume of infarction in the frontal lobe (Spearman's rho= -0.38, *P*=0.024) and frontal cortex (Spearman's rho= -0.47, *P*=0.004).

Figure 5. Regulation of Coagulation and Plasma Proteins after BCAS. Line graphs show the changes in different vascular injury proteins associated with coagulation cascade and BBB functions after BCAS. The most notable changes restored after both limited and continuous EE were vWF and fibrinogen. Calmodulin was increased in EE even in the absence of BCAS and likely protective after subsequent reduced cerebral perfusion and stroke injury. * indicates significant regulation in BCAS-3hrs and BCAS-Full compared to BCAS-Std (FDR adjusted $P<0.001$).

Table 1: Clinicopathological features in elderly subjects with CAD

Variable	Mild ICA stenosis	Moderate ICA stenosis	Severe ICA stenosis
Clinical features			
Number of subjects	12	43	15
% ICA stenosis, mean (range)[#]	44.0 (32.9-51.0)	64.6 (52.3-74.7)	85.0 (75.6-100.0)
Age, years, mean (range)	85.4 (65-93)	86.4 (68-99)	89.4 (83-94)
Gender, number (Female:Male)	7:5	17:26	9:6
Dementia (PSD), number (%)	2 (16.7%)[†]	26 (60.5%)	10 (66.7%)
Total CAMCOG score (/100), mean (range)	85.6 (54-100)**	70.5 (18-96)	70.2 (24-96)
MMSE score (/30), mean (range)	24.7 (15-29)	20.5 (5-30)	19.5 (6-26)
Hypertension, number (%)	6 (50%)	25 (58.1%)	11 (73.3%)
Hyperlipidaemia, number (%)	0 (0.0%)	4 (9.3%)	6 (40.0%)[‡]
Other VRF, (%) (smoking/IHD/Af/DM)	50/8/8/0	61/30/14/5	67/67[¶]/20/13
Pathology markers			
Braak stage, mean (range)	2.3 (1-3)	3.0 (0-6)	3.0 (2-5)
CERAD score, mean (range)	0.6 (0-2)[Ÿ]	1.4 (0-3)	1.0 (0-2)
Vascular Pathology Score, mean (range)[¶]	12.1 (10-15)	13.2 (8-17)	13.4 (8-17)
CAA, moderate-severe (%)	17	20	20

[#]The % ICA stenosis, P=0.000 between all three groups; Age and gender ratio, n.s. between groups; Prevalence of dementia, lowest in the mild ICA stenosis group ([†]P<0.01); Total CAMCOG score, highest in the mild ICA stenosis group ([**]P=0.007); Prevalence of hyperlipidaemia and IHD, highest in the severe ICA stenosis group ([‡]P<0.01 and [¶]P<0.01 respectively); CERAD, lowest in the mild ICA stenosis group ([Ÿ]P=0.022); +75% of demented stroke survivors (PSD) fulfilled pathological criteria for VaD or severe VCI [22, 37]. Braak staging 5-6 were present in 6 moderate and 3 severe ICA subjects. [¶]Vascular Pathology Score [27], n.s. between groups; CAA, moderate-severe (%), n.s. between groups.

Abbreviations: Af, atrial fibrillation; CAA, cerebral amyloid angiopathy; CAMCOG, revised Cambridge Cognition Examination; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; DM, diabetes mellitus; ICA, internal carotid artery; IHD, ischaemic heart disease; MMSE, Mini-Mental State Examination; PSD, post-stroke dementia; VRF, vascular risk factor.

Table 2: Incidence of cerebral infarction in the experimental animals (Sham and BCAS)

	Sham-Std	Sham-3hrs	Sham-Full	BCAS-Std	BCAS-3hrs	BCAS-Full
N	11	11	11	13	10	12
Number with cerebral infarction, number (%)	0 (0.0)	0 (0.0)	0 (0.0)	13 (100)	6 (60)*	8 (66.7)*
Number with cortical infarction, number (%)	0 (0.0)	0 (0.0)	0 (0.0)	9 (81.8)	2 (20.0)$^{¢}$	6 (50.0)$^{¢}$
Number with frontal lobe infarction, number (%)	0 (0.0)	0 (0.0)	0 (0.0)	8 (61.5)	5 (50.0)	6 (50.0)
Number with frontal cortex infarction, number (%)	0 (0.0)	0 (0.0)	0 (0.0)	5 (38.5)	2 (20.0)¶	5 (41.7)

All BCAS-Std animals exhibited cerebral infarction (100%), whereas 60% in BCAS-3hrs and 66.7% in BCAS-Full (*P<0.01); BCAS-3hrs showed the lowest incidence of cortical ($^{¢}P$<0.01) and frontal cortical infarction ($^{¶}P$<0.01); BCAS-3hrs consistently showed lower incidence of infarction amongst BCAS groups. No cerebral infarction was detected in Sham groups.

Abbreviations: BCAS, bilateral common carotid artery stenosis; N, number of animals; Std, standard housing; 3 hrs, limited 3 hours EE; Full, full time EE. (EE, Enriched environment)

Table 3: Differential Regulation of Vascular Injury Associated Proteins after BCAS and Exposure to EE

Protein Description / Gene symbol	# peptides[1]	MW [kDa]	Abundance (log2) ratio (<-1 and >0.6)[2] Sham-3hrs	Sham-Full	BCAS-Std	BCAS-3hrs	BCAS-Full	Brain infarcts[4]	References
Alpha globin 1 / HBA1	13	15.1	0.67	0.74*	1.93	1.77	1.59*	↑ with brain infarcts	[58]
Beta-globin / HBB	1	5.7	0.5	0.53	1.33	1.19	0.61*	↑ neurons under hypoxia	[58]
Fibrinogen beta chain / FGB	14	54.7	0.58	0.25*	1.64	1.47	1.27*	↑ with brain infarcts	[59]
Misato homolog 1 / MSTO1	7	61.2	0.13	0.31*	1.41	0.62*	0.15*	Mitochondria dynamics /aggregation/fusion	[60]
Fibrinogen alpha chain / FGA	10	87.4	0.35	0.16*	1.25	0.97	0.43*	↑ with brain infarcts	[59]
von Willebrand factor / VWF	4	309	1.02	0.71*	2.59	1.85*	1.82*	Pro-thromobotic factor	[61]
Calmodulin / Calm1	14	21.5	1.78	1.71*	1.57	1.54	2*	↑ neuroprotection	[62]
Glycine cleavage system H / GCSH	3	18.6	1.12	1.14	1.76	1.77	1.17*	Controls extracellular glycine/ ↓ infarct size	[63]

Notes: 1. Peptides exclusively identified for the protein. **2**. Abundance log2 ratio in all cases compared to sham. A restrictive cut-off range based on 50% CV was established to indicate significant regulation. All the proteins included in this list shown q-values (FDR adjusted p-values) lower than 0.001. **4.** Potential role(s) of differentially regulated proteins in the apparition/progression of brain infarction based on published reference(s). * indicates significant regulation in sham-EE and BCAS-EE compared to Sham-Std and BCAS-Std respectively.